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(54) Title: COMPOUNDS FOR USE IN THE TREATMENT OF INFLAMMATION

(57) Abstract

Use of a compound comprising an amino acid sequence HFRW in the manufacture of a medicament for inhibition of neutrophil chemoattractant production, inhibition of polymorphonuclear cell (PMN) accumulation, or reduction/treatment of inflammatory response/disease, and/or in the manufacture of an agonist of melanocortin receptor type 3 (MC3–R); wherein the compound is not adrenocorticotrophic hormone $(ACTH)_{1-39}$ or a fragment thereof which activates the production of glucocorticoids.

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COMPOUNDS FOR USE IN THE TREATMENT OF INFLAMMATION

Technical Field

The present invention relates to compounds useful in the treatment of inflammation.

Background of the Invention

Deposition of monosodium urate (MSU) crystals in the joint articular space is the etiological cause of the acute inflammatory condition known as gout or gouty arthritis (Dieppe *et al.*, 1979). Clinically, these inflammatory diseases are associated with edema and erythema of the joints with consequent severe pain. A strong infiltration of leukocytes in the intraarticular and periarticular space is also characteristic of these pathologies. In particular, polymorphonuclear cells (PMN) are the predominant cell type recovered from these inflammatory joints (Terkeltaub, 1992; Dieppe *et al.*, 1979).

Adrenocorticotrophic hormone ($ACTH_{1-39}$) is produced from a larger precursor molecule, which itself is produced by activation of the pro-opio-melanocortin gene. Because ACTH (specifically $ACTH_{1-39}$) acts on the adrenal glands to liberate glucocorticoids such as cortisol (hydrocortisone; for example, in humans) and corticosterone (for example, in rodents), it has always been believed that the effect of ACTH was mediated through the release of these substances which are profoundly anti-inflammatory. However, it has now been found that ACTH possesses a therapeutic efficacy in the treatment of, for instance, gouty arthritis, where it has a beneficial effect over and above that obtained with conventional glucocorticoid therapy (Brandt and Schumaker, 1995).

It has been found that compounds comprising a specific sequence of four amino acid residues in length give rise to a specific anti-inflammatory response which does not involve the production of glucocorticoids and evidence is provided herein that this core tetrapeptide sequence (HFRW; present in, for example, $ACTH_{4-10}$) can inhibit PMN recruitment through an action on the generation of the chemokine KC in experimental

gout. Evidence is also presented that polypeptides incorporating the tetrapeptide sequence are agonists of the melanocortin receptor type 3 (MC3-R). Compounds incorporating the tetrapeptide sequence are therefore useful substrates for a novel approach to anti-inflammatory therapy.

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Summary of the Invention

According to the present invention, there is provided the use of a compound comprising
10 an amino acid sequence HFRW

(i) in the manufacture of a medicament for inhibition of neutrophil chemoattractant production, inhibition of polymorphonuclear cell (PMN) accumulation, or reduction/treatment of inflammatory response/disease, and/or

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(ii) in the manufacture of an agonist of melanocortin receptor type 3 (MC3-R);

wherein the compound is not adrenocorticotrophic hormone $(ACTH)_{1-39}$ or a fragment thereof which activates the production of glucocorticoids.

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The present invention also provides the use of an agonist of melanocortin receptor type 3 (MC3-R) in the manufacture of a medicament for the reduction of inflammation, wherein the agonist is not adrenocorticotrophic hormone $(ACTH)_{1-39}$ or a fragment thereof which activates the production of glucocorticoids.

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Also provided by the present invention is a method of

(i) inhibiting neutrophil chemoattractant production, inhibiting polymorphonuclear cell (PMN) accumulation, or reduction/treatment of inflammatory
30 response/disease, comprising administering to an animal an effective amount of a composition comprising a compound comprising an amino acid sequence HFRW, and/or

(ii) agonism of melanocortin receptor type 3 (MC3-R), comprising administering to an animal an effective amount of a composition comprising a compound comprising an amino acid sequence HFRW;

wherein the compound is not adrenocorticotrophic hormone $(ACTH)_{1-39}$ or a fragment thereof which activates the production of glucocorticoids.

It will be appreciated that background levels of glucocorticoids will be present in a human or animal body. As used herein, reference to a compound that does not activate production of glucocorticoids means that administration of the compound at the dosage employed in the invention does not increase levels of glucocorticoids in a human or animal body. However, increases in glucocorticoid levels may occasionally occur. If so, such increases will be marginal and should preferably not be more than twice, more preferably four times, background levels. Preferably, the compound does not increase levels of any glucocorticoid by more than twice, preferably four times, background levels.

The present invention may employ any compound comprising the amino acid sequence HFRW provided the compound is not adrenocorticotrophic hormone $(ACTH)_{1-39}$ or a fragment thereof which activates the production of glucocorticoids. Preferably, the compound is a polypeptide. The polypeptide may be acyclic or cyclic. The polypeptide may comprise any number of amino acid residues provided that it includes the sequence HFRW and is not $ACTH_{1-39}$ or a fragment thereof which activates the production of glucocorticoids. Preferably, the polypeptide comprises 4-30, preferably 4-20, more preferably 4-12 amino acids. Preferably, the polypeptide comprises the sequence MEHFRWG. Preferably, the polypeptide is a fragment of ACTH, β-melanocortin-stimulating hormone (β-MSH) or a fragment thereof, or MT-II or a fragment thereof.

As used herein, reference to "glucocorticoid" refers to any glucocorticoid compound. Examples of such compounds include cortisol (hydrocortisone) and corticosterone (CCS).

As used herein, reference to "neutrophil chemoattractant" refers to any neutrophil chemoattractant. Preferably, the neutrophil chemoattractant is CXC chemokine KC.

Reference to "inflammatory response/disease" refers to any inflammatory response or disease, preferably gout, gouty arthritis, rheumatoid arthritis, asthma, reperfusion injury or damage, stroke, myocardial infarction, septic shock, or an inflammatory skin disorder, such as psoriasis or eczema.

The present invention also provides the use as described above, wherein the medicament includes one or more pharmaceutically acceptable excipients. Examples of such excipients include phosphate buffered saline (PBS) at, for example, 0.1 M, pH 7.4, $NaHCO_3$ at, for example, 0.2 M and other such physiologically acceptable fluids.

Detailed Description of the Invention

The present invention will now be described, by way of example only, with reference to the accompanying figures, wherein:

Figure 1 illustrates a relationship between the amount of a particular peptide (α-melanocortin-stimulating hormone (α-MSH), β-MSH or $ACTH_{4-10}$) injected into a test animal and the level of PMN migration associated therewith.

Figure 2 illustrates the extent of inhibition of macrophage phagocytosis as measured by flow cytometry.

Figure 3 illustrates, using polymerase chain reaction (PCR) analysis, that melanocortin receptor type 3 (MC3-R) is expressed in murine macrophages.

The glucocorticoid-independent anti-inflammatory effect of peptides incorporating the tetrapeptide HFRW was investigated. Evidence is provided herein that in experimental gout such peptides inhibit PMN recruitment through an action on CXC chemokine KC generation without activating the production of glucocorticoids. Specifically, the effect of the core tetrapeptide HFRW contained within $ACTH_{4-10}$ (MEHFRWG), β-MSH

(AEKKDEGPYRMEHFRWGSPPKD), as well as specific molecules acting as putative agonists or antagonists at melanocortin receptor type 3 (MC3-R), namely MT-II and SHU9119 (Fan *et al.*, 1997), was investigated in an experimental model of MSU crystal-induced inflammation (Getting, *et al.*, 1997).

The compounds used in the present invention are preferably prepared for use as pharmaceuticals. The polypeptides may be administered by any suitable route including oral or parenteral administration. Pharmaceutical compositions which comprise the compounds described typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally or alternatively, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. The polypeptide-containing compositions are preferably administered in combination with pharmaceutically acceptable excipients such as 0.1 M PBS (pH 7.4), 0.2 M $NaHCO_3$ or other such pharmaceutically acceptable fluids.

Typically, the compositions contemplated are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in, for example, liposomes.

Compositions used as pharmaceuticals comprise an effective amount of the compound, as well as any other of the above-mentioned components, as needed. By "effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health, age and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g., non-human primate, primate, etc.), the treating doctor's assessment of the medical situation, and other relevant factors. The amount falls in a relatively broad range that can be determined through routine trials. Typical dosages may fall within the range 0.1-100 mg/kg, preferably 0.5-50 mg/kg, most preferably 1-10 mg/kg.

The compositions contemplated are conventionally administered parenterally, e.g. by injection either subcutaneously or intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule as discussed above. The composition may be administered in conjunction with other anti-inflammatory agents.

As used herein, the term "polypeptide" refers to a polymer of amino acids and is not limited to a specific length of the molecule; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. The polypeptide may be produced by chemical synthesis or by recombinant DNA techniques well known to persons skilled in the art. The term "polypeptide" also includes modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, cyclisations and the like. Included within the definition are, for example, polypeptides containing one or more analogues of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The present invention will now be described with reference the following Examples. It will be appreciated that the following is provided by way of example only and modification of detail may be made without departing from the scope of the invention.

Experimental Protocol

Animals

Male Swiss albino mice (20-22 g body weight) were purchased from Banton & Kingsman (T.O strain; Hull, Humberside), and maintained on a standard chow pellet diet with tap water *ad libitum* using a 12:00 h light/dark cycle. Animals were used 3-4 days after arrival.

In Vivo *Inflammation: Monosodium Urate Crystal (MSU)-Induced Neutrophil Recruitment*

The peritonitis was induced by injection of 3 mg MSU crystals, in 0.5 ml phosphate-buffered saline (PBS; 0.1 M, pH 7.4) (Getting *et al.*, 1997). Six hours later, animals were euthanised by CO_2 exposure, peritoneal cavities washed with 3 ml of PBS containing 3 mM ethylenediaminetetraacetic acid (EDTA) sodium salt and 25 U/ml of heparin. Aliquots of the lavage fluids were then stained with Turk's solution (0.01% crystal violet in 3% acetic acid) and differential counting performed using a Neubauer haemacytometer and a light microscope (Olympus B061). Mononuclear cells and PMN could be easily identified. The large predominance of neutrophils in the PMN population in 6 h-lavage fluids was confirmed in cytospin preparations stained with May-Grunwald and Giemsa, confirming that >95% of PMN were neutrophils.

Drug Treatment

$ACTH_{4-10}$ (Met-Glu-His-Phe-Arg-Trp-Gly, or using the single letter code, MEHFRWG), β-MSH, α-MSH, and MT-II were all from commercial sources (Sigma Chemical Co., Poole, Dorset, UK and Bachem, Saffron Walden, Essex, UK). Scramble $ACTH_{4-10}$ (MGREWFH) was prepared by conventional solid phase chemical synthesis by The Advanced Biotechnology Centre, Charing Cross Westminster Medical School (London, UK) and purified by high liquid performance chromatography. All peptides were more than 95% pure, and were administered subcutaneously at the reported doses 30 min prior to intraperitoneal injection of MSU crystals.

In Vitro *Macrophage Activation*

The selective MC3-R agonist γ2-MSH (Roselli-Rehfuss, *et al.*, 1993; YVMGHFRWDRFG) was tested in an *in vitro* model of inflammatory macrophage activation. Peritoneal cells (>80% macrophages) were collected from untreated mice by lavage and incubated in RPMI-1640 supplemented with 2% FCS and different concentrations of the different agonists (either ACTH, $ACTH_{4-10}$ or γ2-MSH) in a total

volume of 1 ml at 37°C for 15 min. Cells were then diluted to $1x10^6$/ml in Kreb's solution before the addition of 10 µl of the reagent Fc oxyburst red™ (Molecular Probes, Eugene, OR, USA). Uptake of Fc oxyburst red™ complexes by the peritoneal macrophage population was monitored in real time by use of a FACScan (Becton Dickenson, Oxford, UK), which also allowed the quantification of the fluorescence acquired in the FL-3 channel during the 200 s of reaction. Cumulative changes in fluorescence at constant time intervals were then constructed and the area under the curve measured: data are reported as percentage (%) inhibition of macrophage phagocytosis (see Example 4).

Polymerase Chain Reaction (PCR) Analysis

Total RNA was isolated from mouse adherent macrophages with Trizol™ (Gibco BRL, Paisley, UK), with the yield and purity being estimated spectrophotometrically at 260 nm and 280 nm. Total RNA (3 µg) was used for the generation of cDNA using the T-Primed First-Strand kit™ (Pharmacia Biosystems Europe, St Albans, UK). PCR amplification reactions were then performed on aliquots of the cDNA. All PCR reactions were performed using PCR beads™ (Pharmacia) in a final volume of 25 µl using a Hybaid OmniGene thermal cycler (Middlesex, UK). Mouse genomic DNA was used as positive control. The murine melanocortin receptor primer sequences were as follows: *MC1-R,* 5'-GTC-CAG-TCT-CTG-CTT-CCT-GG-3' and 5'-TCT-TCA-GGA-GCC-TGT-GGT-CT-3' (forward and reverse), which amplified a fragment 825 bp in length; *MC3-R,* 5'-GCC-TGT-CTT-CTG-TTT-CTC-CG-3' and 5'-GCC-GTG-TAG-CAG-ATG-CAG-TA-3' (forward and reverse) which amplified a fragment 820 bp in length; *MC4-R,* 5'-ATC-CAT-TTG-CAG-CTT-GCT-TT-3' and 5'-ATG-AGA-CAT-GAA-GCA-CAG-ACG-C-3' (forward and reverse) which amplified a fragment 445 bp in length; *MC5-R,* 5'-ATG-AAC-TCC-TCC-TCC-ACC-CT-3' and 5'-GCA-GTA-GAC-GTT-CTG-AGG-GC-3' (forward and reverse) which amplified a fragment 810 bp in length. The cycling parameters were as follows: initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation (94°C, 45 s), annealing (60°C, 30 s), extension (72°C, 1 min), and a final extension of 72°C for 10 min. Primers for murine GAPDH were also used as positive controls. Amplification products were visualized by

ethidium bromide fluorescence in agarose gels. Images were inverted using the Graphic Converter software (version 2.1) running on a Macintosh Performa 6200 (see Example 5).

Examples

Example 1

In initial experiments, male Swiss Albino mice (28-32 g) were treated subcutaneously (s.c.) with ACTH (20 ng), $ACTH_{4-10}$ (100 μg) or β-MSH (30 μg) 2 h prior to blood collection and measurement of glucocorticoid (corticosterone; CCS) levels by radioimmunoassay in the plasma. In other cases $ACTH_{4-10}$ (10-200 μg), α-MSH (1-30 μg), β-MSH (1-30 μg), MT-II (1-30 μg) or vehicle only (sterile PBS) were given s.c. 30 min before injection intraperitoneally (i.p.) of 3 mg MSU crystals (Getting *et al.*, 1997). Peritoneal cavities were washed with 3 ml of phosphate-buffered saline supplemented with 25 Uml^{-1} heparin 2 h or 6 h later. Differential cell countings of the lavage fluids were done with a Neubauer haematocytometer after staining in Turk's solution. Polymorphonuclear cell (PMN) influx is reported as 10^6 cells per mouse. Release of the chemokine KC was measured in cell-free aliquots of lavage fluids by ELISA (R&D, Abingdon, Oxon, UK). Statistical differences between data (mean ± standard error (s.e.) mean) were assessed by ANOVA (Analysis of Variance).

Whereas ACTH induced an intense release of CCS in the mouse plasma (460 ± 24 $ngml^{-1}$ vs. 42 ± 8 $ngml^{-1}$ in untreated mice; n=6; $P<0.05$), no increase was seen with $ACTH_{4-10}$ or β-MSH (40 ± 5 and 66 ± 12 $ngml^{-1}$, respectively). Injection of MSU crystals caused an intense and long lasting PMN accumulation, with 8.3 ± 0.4 x 10^6 cells per mouse recovered at the 6 h time-point (n=27). Treatment of mice with $ACTH_{4-10}$ inhibited PMN accumulation in a dose-dependent manner, with an approximate ED_{50} of 100 μg per mouse (n=27; $P<0.01$) and a maximal inhibition of 60% at the 200 μg dose. β-MSH was slightly less active, with a calculated inhibition of 46% at the dose of 10 μg per mouse (n=27; $P<0.01$) (higher doses were less effective). Figure 1 illustrates

some of these data which are expressed as percentage (%) of control migration, as measured in vehicle-treated mice. Treatment of mice with 100 μg of a scrambled $ACTH_{4-10}$ peptide (MGREWFH) did not modify MSU-induced PMN migration, whereas the cyclic $ACTH_{4-10}$ derivative MT-II produced again a dose-dependent reduction in the MSU-induced cellular response, with a maximal inhibition of 53% at the dose of 10 μg per mouse (n=6; $P<0.01$).

Example 2

Another series of experiments investigated the effect of ACTH-derived peptides following injection of the MC3-R antagonist SHU9119 (10 μg given i.p. at the same time as the peptides). $ACTH_{4-10}$ (100 μg s.c.) was no longer significantly effective, with a calculated inhibition of PMN influx being reduced from 51% to 17% in the absence or presence of SHU9119, respectively (n=10). Administration of SHU9119 also reduced the inhibitory effect of the agonist MT-II (10 μg s.c.) from 38% to 7% (n=6).

Example 3

The neutrophil chemoattractant CXC chemokine KC was detected in the lavage fluids by ELISA with 5.1 ± 0.4 $ngml^{-1}$ being measured at 2 h post-MSU crystal injection: $ACTH_{4-10}$ reduced this release by 33% (3.4 ± 0.3 $ngml^{-1}$; n=10; $P<0.01$). Much less KC was found at the 6 h time-point (767 ± 85 $pgml^{-1}$, n=17), and $ACTH_{4-10}$ (100 μg), β-MSH (10 μg) and MT-II (10 μg) had an inhibitory effect with 73% (n=18; $P<0.01$), 63% (n=10; $P<0.01$) and 43% reduction (n=6; $P<0.05$), respectively. Treatment of mice with SHU9119 (10 μg i.p.) reduced the inhibitory action of $ACTH_{4-10}$ on KC release to 33% inhibition (n=6; $P<0.05$), and abrogated that of MT-II (9% reduction, not significant).

Example 4

ACTH and $ACTH_{4-10}$ inhibited macrophage phagocytosis as measured by flow cytometry. There was a linear concentration-response curve for both ACTH and $ACTH_{4-10}$ (the latter peptide being almost 1,000 times less potent than the parent

molecule), with almost 40% inhibition at concentrations of 100 ng/ml (22 nM) and 100 μg/ml (104 μM) for ACTH and $ACTH_{4-10}$, respectively. When the selective MC3-R agonist γ2-MSH was tested, again a significant reduction of macrophage phagocytosis was seen, with more than 30% inhibition at the concentration of 104 μM (33 μg/ml) (Figure 2). Importantly, co-addition of the mixed MC3/4-R antagonist SHU9119 (10 μg/ml; equivalent to 9 μM) blocked the inhibitory action of γ2-MSH.

Example 5

All the data shown above indicate that MC3-R and/or MC4-R are responsible for the anti-inflammatory effect of ACTH-derived peptides containing the core sequence HFRW, including the selective MC3-R agonist, γ2-MSH. To discover whether either MC3-R and/or MC4-R were expressed in murine macrophages, PCR analysis was performed. Primers for murine MC1-R, MC3-R, MC4-R and MC5-R were designed and validated using mouse genomic DNA preparations and GADPH mRNA as a positive control (Figure 3). When RNA extracted from peritoneal macrophages (MØ) was used, only the MC3-R band was detected (Figure 3, arrow).

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Claims

1. Use of a compound comprising an amino acid sequence HFRW

(i) in the manufacture of a medicament for inhibition of neutrophil chemoattractant production, inhibition of polymorphonuclear cell (PMN) accumulation, or reduction/treatment of inflammatory response/disease, and/or

(ii) in the manufacture of an agonist of melanocortin receptor type 3 (MC3-R);

wherein the compound is not adrenocorticotrophic hormone $(ACTH)_{1-39}$ or a fragment thereof which activates the production of glucocorticoids.

2. Use according to claim 1, wherein the compound is a polypeptide.

3. Use according to claim 2, wherein the polypeptide comprises 4-30 amino acids.

4. Use according to any preceding claim, wherein the compound comprises an amino acid sequence MEHFRWG.

5. Use according to any preceding claim, wherein the polypeptide is a fragment of ACTH.

6. Use according to claim 5, wherein the polypeptide is $ACTH_{4-10}$ (MEHFRWG).

7. Use according to claim 2, wherein the polypeptide is β-MSH or a fragment thereof.

8. Use according to claim 2, wherein the polypeptide is MT-II or a fragment thereof.

9. Use according to any preceding claim, wherein the neutrophil chemoattractant is CXC chemokine KC.

10. Use according to any preceding claim, wherein the inflammatory response/disease is gout, gouty arthritis, rheumatoid arthritis, asthma, reperfusion injury or damage, stroke, myocardial infarction, septic shock, or a skin disorder.

11. Use according to any preceding claim, wherein the medicament includes one or more pharmaceutically acceptable excipients.

12. Use of an agonist of melanocortin receptor type 3 (MC3-R) in the manufacture of a medicament for the reduction of inflammation, wherein the agonist is not adrenocorticotrophic hormone $(ACTH)_{1-39}$ or a fragment thereof which activates the production of glucocorticoids.

13. A method of

(i) inhibiting neutrophil chemoattractant production, inhibiting polymorphonuclear cell (PMN) accumulation, or reduction/treatment of inflammatory response/disease, comprising administering to an animal an effective amount of a composition comprising a compound comprising an amino acid sequence HFRW, and/or

(ii) agonism of melanocortin receptor type 3 (MC3-R), comprising administering to an animal an effective amount of a composition comprising a compound comprising an amino acid sequence HFRW;

wherein the compound is not adrenocorticotrophic hormone $(ACTH)_{1-39}$ or a fragment thereof which activates the production of glucocorticoids.

14. A method according to claim 13, wherein the compound is a polypeptide.

15. A method according to claim 14, wherein the polypeptide comprises 4-30 amino acids.

16. A method according to any one of claims 13 to 15, wherein the compound comprises an amino acid sequence MEHFRWG.

17. A method according to any one of claims 13 to 16, wherein the polypeptide is a fragment of ACTH.

18. A method according to claim 17, wherein the polypeptide is $ACTH_{4-10}$ (MEHFRWG).

19. A method according to claim 14, wherein the polypeptide is β-MSH or a fragment thereof.

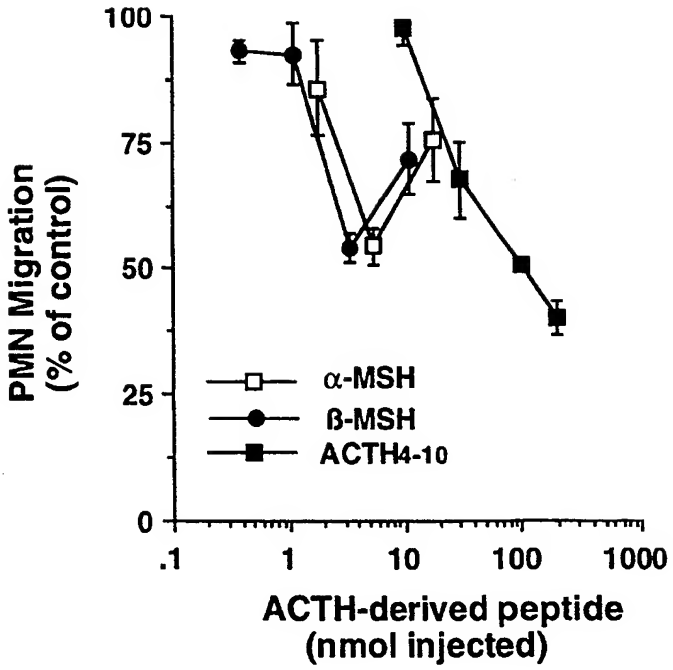
20. A method according to claim 14, wherein the polypeptide is MT-II or a fragment thereof.

21. A method according to any one of claims 13 to 20, wherein the neutrophil chemoattractant is CXC chemokine KC.

22. A method according to any one of claims 13 to 21, wherein the inflammatory response/disease is gout, gouty arthritis, rheumatoid arthritis, asthma, reperfusion injury or damage, stroke, myocardial infarction, septic shock, or a skin disorder.

23. A method according to any one of claims 13 to 22, wherein the composition includes one or more pharmaceutically acceptable excipients.

Figure 1


100
75
50
25
0
PMN Migration
(% of control)
α-MSH
ß-MSH
ACTH4-10
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1
10
100
1000
ACTH-derived peptide
(nmol injected)

Figure 2

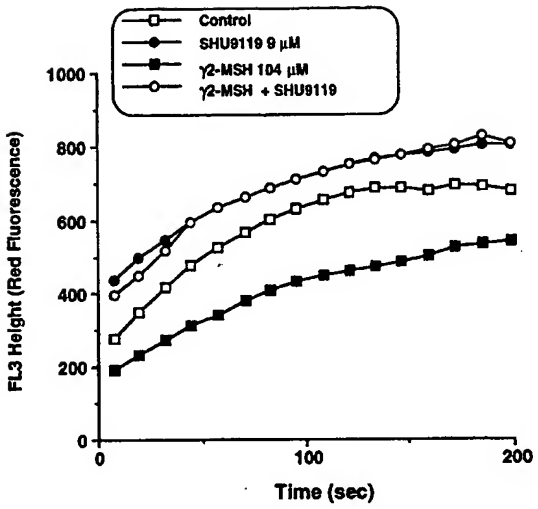
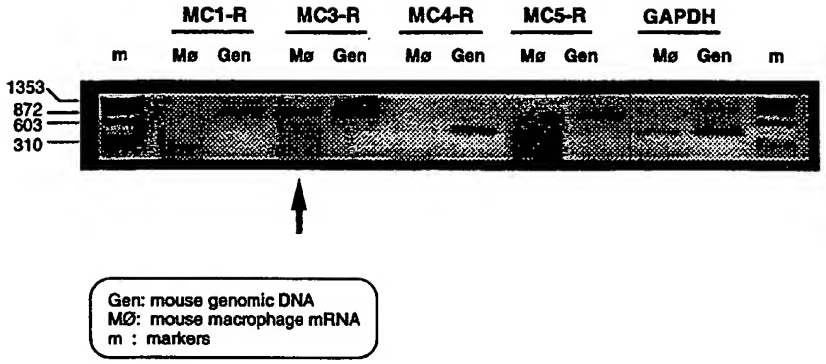

Control
SHU9119 9 µM
γ2-MSH 104 µM
γ2-MSH + SHU9119
FL3 Height (Red Fluorescence)
1000
800
600
400
200
0
0
100
200
Time (sec)

Figure 3


MC1-R
MC3-R
MC4-R
MC5-R
GAPDH
m
Mø Gen
Mø Gen
Mø Gen
Mø Gen
Mø Gen
m
1353
872
603
310
Gen: mouse genomic DNA
MØ: mouse macrophage mRNA
m : markers